SUCCINIC ACID AS A BYPRODUCT IN A CORN-BASED ETHANOL BIOREFINERY

Award No. DE-FG36-06GO86031
MBI Project No. 06-1575

FINAL REPORT

July 1, 2006 through November 30, 2007

Submitted to
U.S. Department of Energy – Golden Field Office
1617 Cole Boulevard
Golden, CO 80401-3305
ATTN: Gene R. Petersen

Submitted by
MBI INTERNATIONAL
P.O. Box 27609
Lansing, MI 48909-0609

Contact:

Project Director :
Dr. Susanne Kleff
Telephone: 517.336.4677
E-mail: kleff@mbi.org

Business Contact:
Catherine Johnson
Telephone: 517.336.4645
E-mail : johnson@mbi.org

MBI INTERNATIONAL
Final Report
FOR THE PERIOD JULY 1, 2006 – NOVEMBER 30, 2007
SUCCINIC ACID AS A CO-PRODUCT IN A CORN-BASED ETHANOL BIO-REFINERY

TABLE OF CONTENTS

1.0 Executive Summary

MBI endeavored to develop a process for succinic acid production suitable for integration into a corn-based ethanol biorefinery. The project investigated the fermentative production of succinic acid using byproducts of corn mill operations.

The fermentation process was attuned to include raw starch, endosperm, as the sugar source. A clean-not-sterile process was established to treat the endosperm and release the monomeric sugars. We developed the fermentation process to utilize a byproduct of corn ethanol fermentations, thin stillage, as the source of complex nitrogen and vitamin components needed to support succinic acid production in *A. succinogenes.* Further supplementations were eliminated without lowering titers and yields and a productivity above 0.6 g l^{-1} $hr^{-1}$was achieved.

Strain development was accomplished through generation of a recombinant strain that increased yields of succinic acid production. Isolation of additional strains with improved features was also pursued and frozen stocks were prepared from enriched, characterized cultures.

Two recovery processes were evaluated at pilot scale and data obtained was incorporated into our economic analyses.

2.0 Project Activities

2.1 Project Description

MBI has developed a batch fermentation process for the production of succinic acid using *Actinobacillus succinogenes*. The organism exhibits several advantageous features such as high glucose tolerance, no significant product inhibition, good osmotolerance and simultaneous use of pentose and hexose sugars. Succinic production has the environmental advantage of CO_2 fixation.

The economic model showed that the cost of succinic production was directly affected and had high sensitivity to the cost of the fermentable carbon source, glucose. Use of a lower cost carbon source such as raw starch has the potential to improve the process economics.

The current fermentation process requires addition of complex medium components to achieve high levels of succinic acid production. Corn based bio-refineries produce multiple by-product streams, some of which are ultimately used as low cost animal feeds. These streams contain nitrogen and vitamins from corn and yeast ethanol fermentations and were tested to determine if they can supply some nutrients needed in succinic acid production. Furthermore, a detailed, systematic analysis to examine the specific auxotrophies of the organism under production conditions was initiated. Although defined minimal media are known for *A. succinogenes* (Guettler, 1996, McKinley, 2005) and closely related organisms these media allow only limited growth and are not suitable for high productivity; however, they were used as starting point to identify critical components stimulatory for growth and succinic production. Molecular biological methods, tools and *A. succinogenes* genome information have been established that would allow identification of missing gene products and augmentation through heterologous gene expression in the organism.

Competitive production of commodity chemicals by fermentation is very cost restricted. Although the productivity and titers seen with our current organism are unsurpassed, the costs associated with neutralization and complex medium components restrict the market potential of

succinic acid. Further strain enhancement is feasible but will have limits. We propose to isolate a novel organism from the same environment that harbored *A. succinogenes*, but improving the selection scheme to avoid the complex medium requirements.

Economic modeling of succinic acid production costs requires input from the fermentation and recovery steps of the process. MBI International has investigated two recovery processes for succinic acid from fermentation broth at pilot scale, direct acidification and ion-exchange. Improved material balances, recovery yields and product purity need to be established to complete the economic model.

The goal of this project was to develop an economical fermentation process for the production of succinic acid by fermentation using low-cost corn products. This was attempted using three approaches:

Objectives:

- Enhance succinic acid production in *A. succinogenes* from low-cost raw starch and corn based bio-refinery by-products as feedstock including targeted strain alterations in gene expression.
- Isolation of a novel organism from the bovine rumen capable of high level succinic acid production following a previously successful approach, but attempting to reduce complex medium requirements.
- Evaluate a downstream recovery process for succinic acid with material balances, recovery yields and product purity to gather input for an economic model to project succinic production costs at a 50MM lbs y^{-1} scale.

Tasks:

Task A. Utilization of corn bio-refinery products for succinic acid production

Task A.1 Raw starch fermentations

Task A.2 Fed-batch process development

Task A.3 Medium additions to enhance performance

Task B. Strain improvements through targeted gene alterations and isolation of an improved hyper-acid producer from the bovine rumen.

Task B.1 Strain improvements through targeted gene expression

Task B.2 Isolation of an improved hyper-acid producer

Task C. Investigation of a downstream recovery process

Task C.1 Perform fermentations at the 100 L scale

Task C.2 Assess feasibility of various methods to enhance recovery of succinic acid.

Deliverables

1. A technical report, including economic analysis, for production of succinic acid using raw starch as carbon source.
2. Report on status of isolation of hyper-acid producer. Established bank of isolates.
3. Report detailing processes, mass balance, and yield and purity of the succinic acid produced.

2.2 Task A: Fermentations with A. succinogenes

2.2.1 Introduction

MBI had demonstrated that succinic acid production from corn raw starch is possible, using a rich medium supplemented with yeast extract. We also showed that use of high concentrations of yeast extract is not cost-effective for the production of commodity chemicals. We tested, if and to what extent low cost by-products such as stillage can replace yeast extract. Fermentations were monitored for growth, product titers and yields. The need for further supplements was assessed. Although the strict growth requirements of *A. succinogenes* and related organisms have been established, they do not support the growth and productivity needed for economic production, but were used as the basis for additions to be tested. Categories of interest were peptide sources, vitamins, nucleotide co-factors. We planned on utilizing the available genome data to establish existence and missing links in biosynthetic pathways, similar to those seen in close relatives such as *M. succiniciproducens* (Hong, 2004). Strain improvements through gene-over-expression were incorporated into these efforts to bypass deficiencies, where possible.

2.2.2. Materials and Methods

Microorganism and Cultivation. Actinobacillus succinogenes FZ45 and other derivatives are stable bacterial variants made by chemical selection of the parent strain *Actinobacillus sp.* 130Z (ATCC 55618, Guettler et *al*., 1999; Guettler et *al.* 1998 US Patent 5,723,322).

A. succinogenes FZ fermentations were performed in 5 l fermentors essentially as described in Guettler, et *al.* 1999. For MFA analysis the starting glucose concentration was 80 g/l, and the medium was supplemented with yeast extract to allow for lower inoculum and monitoring of extrended growth. The pH was maintained at 7.0 with $Mg(OH)_2$. Standard fermentations were performed in a similar medium in which the yeast extract was omitted. The pH was maintained with a slurry of $Mg(OH)_2$ and Na_2CO_3 , inoculum volume 5%. Small-scale seed vials were prepared with 2-4g of $MgCO_3$ sterilized twice with 1ml water. Medium additions to 50 ml final volume were made as for MFA fermentations. Vials were flushed three times with CO_2 and incubated with gentle shaking at 37ºC. Fermentations using corn mill by-products as medium component were supplemented with inorganic phosphate, dextrose starting concentrations ranged from 75-95g/l. Seed fermentors were supplemented with yeast extract and corn steep liquor. Other additions were made as described in the text.

E. coli was grown in shake flasks in LB medium with/without the appropriate antibiotic at 37ºC, shaking at 200 rpm in a New Brunswick gyrotory shaker. Strain DH5α was used for all cloning purposes.

Substrate and product analyses. Succinic acid, glucose, lactic acid, pyruvate, ethanol, and formic acid concentrations were determined by reverse phase high pressure liquid chromatography (HPLC) using a Waters 1515 isocratic pump with a Waters 717 Auto sampler and a Waters 2414 refractive index detector set at 35^{o}C. The HPLC system was controlled, data collected and processed using Waters Breeze software (version 3.3). A Bio-Rad Aminex HPX-87H (300mm x 7.8mm) column was used with a Cation H guard column held at 55^{o}C. The mobile phase was 0.021N sulfuric acid running at 0.5 ml/min. Samples were filtered through a 0.45 μm filter, and 5.0 µl were injected onto the column. Run time was thirty minutes.

A mass flow controller (Brooks model 5850I) was used to monitor and supply CO_2 to the fermentor sparging system at 100 ml/min. A mass flow meter (Brooks model 5860I) was used

to measure CO_2 exiting the fermentor by way of the exhaust condenser system. The two CO_2 flow meters were connected to a computer via a 4-20ma Bio-Command Interface. The BioCommand Plus Bioprocessing software logs the inlet and outlet CO_2 flow every 60 seconds. The rate of CO_2 consumption (ml/min) was expressed as the difference between the inlet and outlet rates during any given minute ($CO_{2use} = CO_{2in} - CO_{2out}$). The moles of CO_2 consumed were calculated using the Ideal Gas Law, (22.4 liters/mole). The mass flow meters were calibrated by the manufacturer for CO_2 and their precision is 1% of full scale or 2 ml/m. The fermentation set-up was monitored for gas leaks, by mixing 5% hydrogen into the CO_2. Hydrogen leaks are detected using a Tif8800 CO/Combustible Gas analyzer.

2.2.3. Results and Discussion

Fermentations using raw starch, endosperm

We demonstrated corn mill by-products (CMBP) can provide all the needed components to support fermentations with good succinic acid productivity, when using refined glucose. In the endosperm (ES) fermentations, starch was saccharified in the medium prior to minor additions of phosphates. In the first experiment, the ES starch underwent liquefaction and saccharification following published procedures. Prior to starting the succinic fermentation, the temperature was raised to reduce the microbial load entering with CMBP and ES. Figure 1 shows a successful fermentation with endosperm as the source of glucose in a CMBP medium supplemented with 2g/L protein hydrolysate. The fermentation produced just over 70g/L succinic acid with a 96% yield in 84 hours. At 118 hours the succinic concentration was 72 g/l and the residual glucose was 2.81 g/L. There was no consumption of the succinic acid product, or residual glucose by contaminating species in this clean but not sterile (CNS) fermentation.

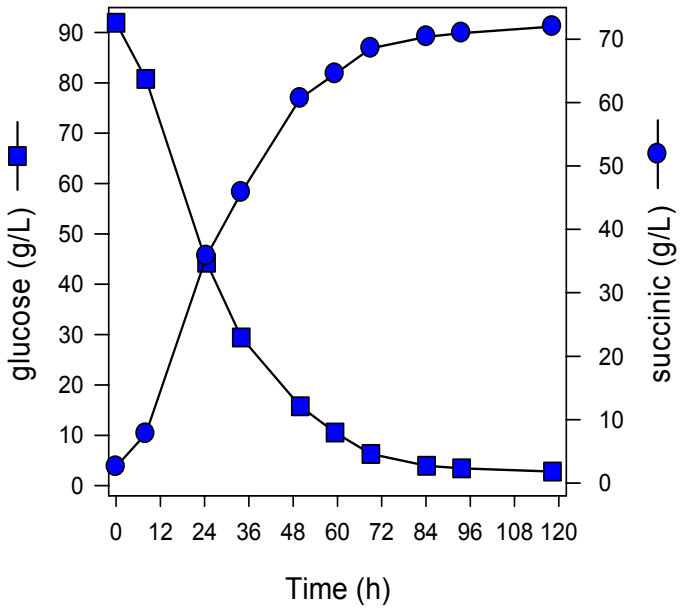

Figure 1. *A. succincogenes* FZ53 fermentation 225-07. The medium contained CMBP, glucose derived from hydrolyzed ES, and was held at elevated temperature prior to fermentation. Protein and other minor additions were added.

Subsequent efforts were spent on reducing the amount of protein hydrolysates as a production medium supplement. In clean sugar fermentations the omission of protein hydrolysates achieved the same titers compared to those with added protein hydrolysates, but the total fermentation time increased by about 24 hours (Figure 3). Several processing modes were tested that would allow some pasteurization of ES and CMBP. The length of heating time, temperature of the heating, and the process point of heating, i.e. before or after saccharification, were tested and a best mode of operation was established. After cooling, pH adjustment, and addition of minor medium components the succinic acid fermentation was started. Fermentation 1207-07 used ES and CMBP with the established treatment scheme. Even though it contained microorganisms other than *A. succincogenes* the fermentation produced 65 g/l of succinic in 73 hours (Figure 2), and the succinic yield at 85 hours was 92%. The performance of fermentation 1207-07 compares favorably with fermentation 225-07 (Figure1), which was supplemented with protein hydrolysate.

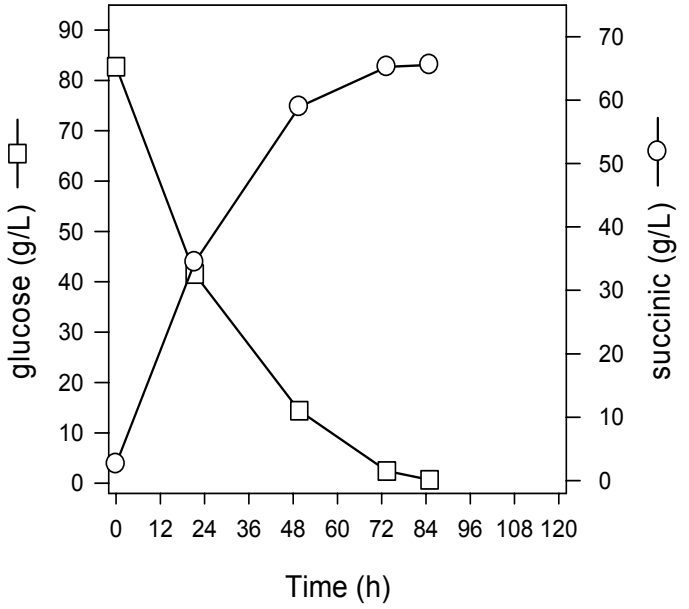

Figure 2. *A. succincogenes* fermentation 1207-07. The medium contained CMBP, and glucose derived from hydrolyzed ES. The CMBP and ES were heated prior to saccharification to reduce the number of contaminating microorganisms while minimizing the production or release of inhibitory compounds that affect succinic production.

The addition of the antibiotics was also evaluated. They reduced the amount of contaminants and the onset of their growth, but could not completely abolish contamination towards the end of the fermentation.

Economic modeling of the fermentations assumed that the treatment generates a sugar stream at a cost of 6 cents/lbs (Licensee personal communication).

Develop a fed-batch process for succinic acid production

The development of a fermentation process using thin stillage and endosperm had confirmed a previous made observation that the primary stimulatory factor of productivity was nitrogen. We were also very aware of the fact that protein hydrolysates enhance succinic production but are cost prohibitive. We demonstrated in small vial fermentations that addition of inorganic

compounds showed a slight enhancement in final succinic titers. A fed-batch fermentation at 5L scale delivering these compounds throughout the fermentation was performed, but failed to increase titers, yields or productivity.

A. succinogenes fermentations are characterized by a very short growth phase, coupled with high productivity, a production phase with high productivity, and a production phase with declining productivity associated with decline of biomass. As a potential way to maintain productive biomass for a longer time we tested the performance of fermentations with a feed of fresh cells along with medium into a finishing tank. We achieved higher titers of 70 g/l, compared to 60-65g/L for standard batch fermentations. In addition, a bolus of glucose was added towards the end of the fermentation and was readily consumed. This indicated the potential for further and prolonged activity of the biocatalyst. We repeated this fermentation and increased the glucose feed towards the end as a test of the cell's potential. However, in this run the glucose was not completely consumed and the fermentation did not finish. We assumed that the seed train was not as potent as seen previously. Since the set-up even at lab scale was cumbersome, and we saw only a small improvement in titer, we abandoned this approach.

Enhance succinic acid production through medium additions
To facilitate the integration of succinic acid production into a corn based ethanol bio-refinery the use of by-products generated on site is expected to benefit the economics. Our organism requires complex medium components to exhibit good productivity and we had previously developed processes that utilize yeast extract, corn steep liquor, CSL, or liquid feed syrup, LFS, as medium components. The latter are products from the corn wet milling industry. The majority of ethanol is produced by dry milling operations, which also generate by-products often sold as part of animal feed. We tested if these CMBPs could support succinic fermentations and if additional supplements were needed to achieve productivity >0.6 g/l hr, and titers around 60g/l. Initial fermentations using CMBP at various levels and *A. succinogenes* isolate FZ45 showed only limited growth and poor succinic production. We screened other FZ derivatives from our *A. succinogenes* FZ strain bank and identified one which showed comparable succinic production as seen in other growth media.

A detailed analysis was performed to establish the amount of CMBP needed to support succinic production, and identification of the nature of additional, production-enhancing supplements. Clearly, more CMBP as medium component yielded higher amounts of succinic acid. These fermentations were performed using clean sugar syrup. Further investigations tested additions of amino acids, vitamins, and minerals to define the important components provided or missing in CMBP. These experiments were performed low-strength CMBP medium. Addition of most components had no effect, indicating that sufficient amounts of these are available in CMBP. The biggest improvement came from addition of amino nitrogen, indicating that those compounds were in limiting supply. We attempted to identify the specific missing components, but these fermentations were inconclusive. We assume that the control and feed-back mechanisms masked or interfered with the identification of specific deficiencies. Fermentations such as 125-07 had a productivity of ~1.4 g/ l hr and a final titer of ~70 g/l succinic in a supplemented CMBP medium (Figure 3).

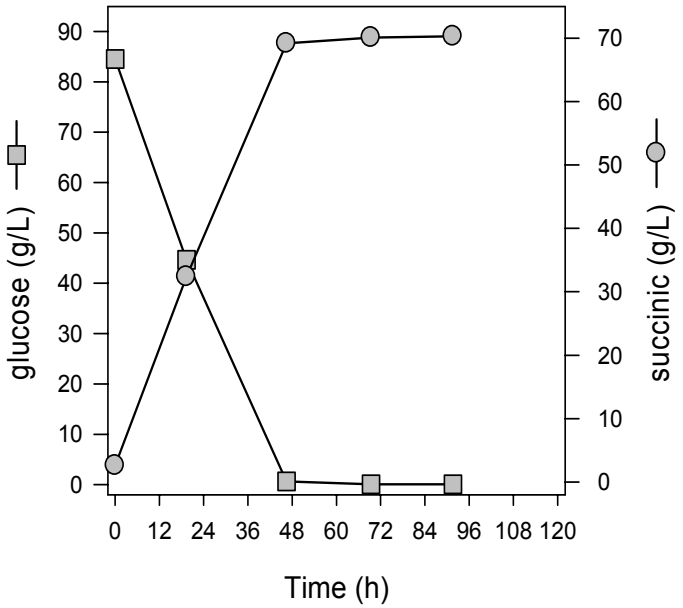

Figure 3. *A. succincogenes* fermentation 125-07. The medium contained CMBP, glucose, and 2g/L of supplements. The fermentation was started with a 5% inoculum.

Economic analysis of these fermentations indicated that 2g/l protein hydrolysate was cost prohibitive despite the higher succinic productivity. Seed development and changes in the inoculum volume were investigated as a means to lower protein hydrolysate required in the production medium. The current process uses a seed medium with high CMBP supplemented with CSL and yeast extract. The production medium contains high CMBP and no other complex nitrogen sources. The fermentations showed productivities of 0.75 g/l hr, titers of ~70g/l, yields of 93% (Figure 4).

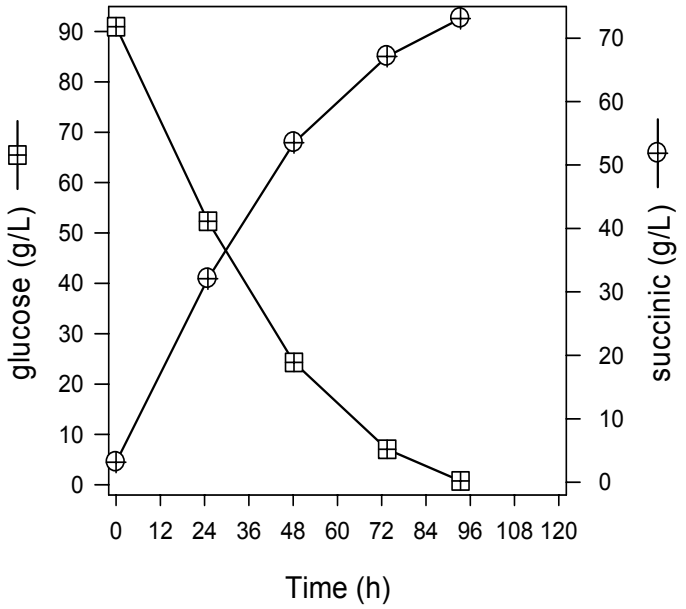

Figure 4 *A. succincogenes* fermentation 1017-07. The medium contained high CMBP, glucose, and no other complex nitrogen source.

2.2.4. Conclusions

Under this task we developed a fermentation process for the production of succinic acid utilizing almost exclusively by-products of corn dry milling operations in the production stage. Although MBI had been working with corn milling by-products such as CSL and LFS in succinic fermentations, it was unexpected to observe the poor performance of our previously favored isolate, FZ45, in CMBP medium. Furthermore, although all FZ derivatives of *A. succinogenes* were derived from the same strain 130Z, and further selected following the same scheme, their environmental tolerances differ. The metabolism of the currently used isolate differs in the production of slightly higher level of acetate compared to FZ45. This by-product decreases the succinic yield slightly, 95% compared to 99% for FZ45, but the presumed favorable energetics associated with its production may make it less susceptible to some external factors present in CMBP. The performance of this isolate in CMBP medium could also be an indication of considerable osmo-tolerance of this isolate.

Over the course of this grant many different batches of CMBP were used and assessed for their ability to support succinic acid production. Storage of CMBP was only possible for a limited time period, and shipping introduced occasional microbial growth, as evident by the amount of lactic acid in some batches. This will not be a consideration in a biorefinery production setting.

2.3 Task B: Second Generation Organisms

2.3.1 Introduction

Under Task A MBI had established a fermentation process using thin stillage as the sole complex nitrogen source in the fermentation. A detailed analysis of defined nitrogen components that further enhances productivity in this medium background was not further pursued, since the productivity seen without any supplementation was comparable to previously

used media containing liquid feed syrup or corn steep liquor. Additional limiting factors of the fermentation process were investigated.

Although *A. succinogenes* shows good productivity, titer and yield our economic modeling had identified the major cost factors and some practical implementation hurdles seen with the current strain and process. One challenge is the use of inexpensive complex medium components, which can affect the recovery yields and product purity, or demand additional, costly recovery steps. In addition, the current magnesium base used for neutralization of the product during fermentation is not commonly used in current industrial fermentation processes. Engineering a strain to by-pass broad and general traits can be very cumbersome and time-consuming. Similarly, adaptation of a strain to conditions that are different from those used in its isolation has a high likelihood of failure. Instead, we decided to attempt isolation of a novel strain. The rumen represents an environment rich in microorganism capable of fermenting a variety of carbon sources and converting them to propionic, succinic and acetic acids. It also provides a CO_2 rich atmosphere, which is conducive for the anaerobic production of succinic acid. The successful isolation of our currently used organism, *A. succinogenes*, and *M. succiniciproducens* (Lee, 2002) testifies that the rumen is a good source for the isolation of succinic acid producers. A repeated isolation attempt would incorporate many features previously used (Guettler, 1999), but attempt to lower the nutritional requirements and lower the overall Mg concentrations in the isolation scheme to obtain an organism that is less dependent on Mg for optimal production.

We knew that biomass of *A. succinogenes* diminishes over the course of the fermentation. We planned on assessing if addition of proteins, vitamins, co-factors can prolong the stationary phase of the catalyst, and prevent apoptosis. Alternatively, cell death could be caused through accumulation of toxic metabolites (Booth, 2003). Literature searches on identified toxic metabolites in other organisms will guide attempts to either prevent accumulation or identify enzymatic activities that would degrade the metabolites. Metabolic alterations would be accomplished through over-expression of remediating gene products.

2.3.2 Materials and Methods

Genetic Manipulations. Recombinant DNA manipulations generally followed methods described by Sambrook, 1990. Plasmid DNA was prepared with the alkaline lysis method. Typical resuspension volumes for multicopy plasmids extracted from 1.5ml cultures were 50μl. Larger DNA preparation used the Qiagen Plasmid Purification Midi and Maxi kit according to the manufacturer's instructions. Restriction endonucleases, molecular weight standards, and prestained markers were purchased from NE Biolabs and Invitrogen and digests were performed as recommended by the manufacturer, except that 2-5-fold excess of enzyme was used. DNA was analyzed on agarose gels in the presence of ethidium bromide. DNA was extracted from agarose gels and purified using the Qiagen gel extraction kit according to the manufacturer's instructions. Plasmid DNA was dephosphorylated using rAPID phosphatase (Roche) in combination with restriction digests. Phosphatase was heat inactivated at 70°C for 2min. Ligations were performed using a 3-5-fold molar excess of insert to vector DNA in 15-20μl reaction volumes and 1μl of T4 DNA Ligase (NE Biolabs) for 0.5 – 3 hours at room temperature. *E. coli* transformations were carried out using "library efficiency competent cells" purchased from Invitrogen, following the manufacturer's instructions. Transformations using ligation mixes were plated without dilutions on standard LB plates (Sambrook, 1990), containing the appropriate antibiotic. PCR amplifications were carried out using the Sigma AccuTaq enzyme and reaction set up. Primer designs were based on published sequences (NCBI database) with added restriction recognition sites and analyzed for dimer and hairpin formation

and melting temperature using the Vector NTI program. All primers were ordered from the Michigan State University Macromolecular Structure Facility. PCR amplifications were carried out in an Eppendorf Gradient Master Cycler. Starting annealing temperatures were determined using the vector NTI program for each primer pair.

Plasmid pJR762.73
The gene balancing reducing power from *E. coli* was PCR amplified from BL21 genomic DNA. XhoI restriction sequences were included to facilitate cloning. Localization of the resulting 1.5-kb fragment into the SalI site of pJR762.55, described in previous reports, resulted in pJR762.73.

Plasmid pREB830.55. The corresponding open reading frame, ORF, from *A. succinogenes* was PCR amplified. The product was digested with SalI and BamHI and inserted into the SalI-BamHI sites of pJR762.55.

Plasmid pREB830.60. The entire *A. succinogenes* gene including its promoter were PCR amplified cut with SalI and BamHI and inserted into the same sites of pJR762.47.

Transformation of A. succinogenes. A. succinogenes competent cells for electroporation were prepared by growing cells in TSB medium. Excess carbonate was removed by centrifugation at 420xg; cells were spun down at 6,500xg, washed twice with sterile water and twice with 10% v/v glycerol and resuspended in 0.01x the original culture volume of 10% glycerol. Cells were flash frozen and stored at -80°C. 40 μl of prepared cells were used for electroporation, using 0.1 cm cuvettes and a BioRad GenePulser with settings of 400W, 25mF, 1.8kV. Following electroporation, 1ml room temperature TSB medium was immediately added to the cuvette and incubated at 37°C for 1 h. The cell solution was plated on TSB agar plates containing antibiotic.

Microorganism and Cultivation. Actinobacillus succinogenes FZ45 and other derivatives are stable bacterial variants made by chemical selection of the parent strain *Actinobacillus sp.* 130Z (ATCC 55618, Guettler et *al*., 1998; Guettler, et *al.* 1996, US Patent 5,573,931).

Microorganism and Cultivation. Actinobacillus succinogenes FZ45 and other derivatives are stable bacterial variants made by chemical selection of the parent strain *Actinobacillus sp.* 130Z (ATCC 55618, Guettler et *al*., 1999; Guettler et *al.* 1998 US Patent 5,723,322).

A. succinogenes FZ fermentations were performed in 5 l fermentors essentially as described in Guettler, et *al.* 1999. For MFA analysis the starting glucose concentration was 80 g/l, and the medium was supplemented with yeast extract to allow for lower inoculum and monitoring of extrended growth. The pH was maintained at 7.0 with $Mg(OH)_2$. Standard fermentations were performed in a similar medium in which the yeast extract was omitted. The pH was maintained with a slurry of $Mg(OH)_2$ and Na_2CO_3 , inoculum volume 5%. Small-scale seed vials were prepared with 2-4g of $MgCO_3$ sterilized twice with 1ml water. Medium additions to 50 ml final volume were made as for MFA fermentations. Vials were flushed three times with CO_2 and incubated with gentle shaking at 37ºC. Fermentations using corn mill by-products as medium component were supplemented with inorganic phosphate, dextrose starting concentrations ranged from 75-95g/l. Seed fermentors were supplemented with yeast extract and corn steep liquor. Other additions were made as described in the text.

E. coli was grown in shake flasks in LB medium with/without the appropriate antibiotic at 37ºC, shaking at 200 rpm in a New Brunswick gyrotory shaker. Strain DH5α was used for all cloning purposes.

Substrate and product analyses. Succinic acid, glucose, lactic acid, pyruvate, ethanol, and formic acid concentrations were determined by reverse phase high pressure liquid chromatography (HPLC) using a Waters 1515 isocratic pump with a Waters 717 Auto sampler and a Waters 2414 refractive index detector set at 35^{o}C. The HPLC system was controlled, data collected and processed using Waters Breeze software (version 3.3). A Bio-Rad Aminex HPX-87H (300mm x 7.8mm) column was used with a Cation H guard column held at 55^{o}C. The mobile phase was 0.021N sulfuric acid running at 0.5 ml/min. Samples were filtered through a 0.45 μm filter, and 5.0 µl were injected onto the column. Run time was thirty minutes.

A mass flow controller (Brooks model 5850I) was used to monitor and supply CO_2 to the fermentor sparging system at 100 ml/min. A mass flow meter (Brooks model 5860I) was used to measure CO_2 exiting the fermentor by way of the exhaust condenser system. The two CO_2 flow meters were connected to a computer via a 4-20ma Bio-Command Interface. The BioCommand Plus Bioprocessing software logs the inlet and outlet CO_2 flow every 60 seconds. The rate of CO_2 consumption (ml/min) was expressed as the difference between the inlet and outlet rates during any given minute ($CO_{2use} = CO_{2in} - CO_{2out}$). The moles of CO_2 consumed were calculated using the Ideal Gas Law, (0.88694 liters ÷22.4 liters/mole = 0.0396 moles). The mass flow meters were calibrated by the manufacturer for CO_2 and their precision is 1% of full scale or 2 ml/m. The fermentation set-up was monitored for gas leaks, by mixing 5% hydrogen into the CO_2. Hydrogen leaks are detected using a Tif8800 CO/Combustible Gas analyzer.

Metabolic modeling and metabolic flux analysis. The metabolic network of *A. succinogenes* was constructed in FluxAnalyzer 4.3 (Steffen Klamt, Max-Planck-Institute, Germany). The accessory MATLAB including an optimization toolbox was purchased from MathWork Inc. The metabolic network was based on previous work (van der Werf et *al*., 1997) and enzyme assay results described under previous funding periods (see report under DOE/GO12001-FTR04). The major components of the network include glycolysis, pentose phosphate pathway (PPP), the reductive tricarboxylic acid cycle, Phosphoenolpyrucate (PEP) carboxykinase, citrate lyase, pyruvate catabolism and acetyl-coA catabolism. NADH and NADPH were considered to be the same in this metabolic network. For uptake of carbon sources, no phosphoenolpyruvate:carbohydrate phosphotransferase (PTS) system was considered because most of the PEP was used by PEP carboxykinase to produce succinic acid through the reductive TCA cycle. Altogether, there are 27 reactions and 20 important intermediates (see report under DOE/GO12001-FTR04).
Metabolic flux analysis was performed in a FluxAnalyzer 4.3 to calculate the intracellular metabolite fluxes. From the HPLC measurements and the measured flow rates of influent and effluent, the specific consumption rates of carbon source and CO_2, and the specific production rates of succinic acid, acetic acid, pyruvate and ethanol were calculated. The consumption rate of 6 C-source such as glucose was normalized to 100. To obtain theoretical yield for succinic acid production from different carbon sources, the succinic acid production was used as a target function to be optimized in FluxAnalyzer under the constraint of reducing power balance.

Enrichment cultures
All enrichment cultures were set up in 500 ml Multigen vessels, equipped with pH control, temperature control, and a CO_2 sparge rate of ~0.025 v.v.m. Feed rate was varied from 0.4ml/min to 0.8ml/min. Three different media were used, CMBP medium without any other additions and a pH adjusted to near neutral, Middelbrook medium (DIFCO), RCCPM (Jablonski,

P. E. et *al*., 1996), a minimal medium for growth of *Pasteurella multocida*, with some modifications. The carbon sources used were xylose, glucose, arabinose, glycerol, and glycerol from biodiesel, obtained from "Kramer Biodiesel", density of 1.2g/ml. Cultures were analyzed for products by HPLC as described above daily. Glycerol stocks of enriched cultures were frozen and saved when a cleaner product profile was noticed and remained unchanged for 72hrs.

2.3.3 Results and Discussion

Development of a chromosomal Integration system in A. succinogenes

Systems for chromosomal integration in related organism were assessed in the literature. A disruption vector was constructed, harboring the following features: the sequence of an *A. succinogenes* gene, the insertion of an antibiotic resistance within this gene's coding region under a recognized promoter, a vector backbone that cannot replicate in *A. succinogenes*. The antibiotic resistance insertion into this gene was accompanied by a partial gene deletion. Transformations with supercoiled and linearized forms of this vector required several attempts to obtain a few antibiotic resistant colonies. In order to determine the integrations sites we performed PCR amplifications using genomic DNA from these transformants and the parental control as template. Three sets of primers were used: (1) a pair complementary to the *A. succinogenes* gene target, (2) a primer pair corresponding to the antibiotic resistance gene, and (3) a pair with one primer being complementary to the gene and the other complementary to the antibiotic resistance gene. The predicted lengths of each PCR products differed and would allow to make the distinction between disruption of the chromosomal locus and random integration associated with duplication of the locus. The PCR using primer set 1 showed the same size band as the untransformed, parental control, suggesting that the target locus was still intact and not disrupted. However, we did not see a second, larger band indicating the disrupted locus. It is possible, albeit unlikely, that the undisrupted, original locus would be amplified preferentially, since it gives rise to a smaller PCR product, which is generally easier to obtain. But we did not see any indication of a larger band. The other two primer pairs showed PCR product of sizes that indicated an integration event, when using genomic DNA from a transformed strain. In addition, growth of the transformed strains without antibiotic selection over > 30 doublings still showed antibiotic resistance upon plating on selective plates. Since the plasmid backbone is an *E.coli* vector, pUC, that cannot replicate in *A. succinogenes*, it is unlikely that it could have been maintained in the cells for such extended growth periods. Further PCR analyses were performed in which one primer annealed downstream of the gene locus according to the genome contig in combination with the primers annealing to the antibiotic resistance gene. But we obtained no PCR product. However, this negative result is not conclusive, and a perfect control for this PCR is not possible. In conclusion, the disruption construct DNA appeared to have integrated, but without disrupting the original locus.

In an alternative approach, since chromosomal integration might not be successful, or not produce the desired gene copy number, we determined the stability of our plasmid vector without selective pressure, i.e. presence of antibiotic. The presence of one of our constructs, described in Table 1, pREB830.60, can be recognized by the yield improvements they confer to the recombinant strain. We maintained the strain in growth phase over approximately 50 generations. Succinic acid yields were compared to a vector control for cultures grown in the presence and absence of antibiotic. The results indicated that the plasmid is stable over approximately 30 cell divisions without selective pressure.

Plasmid stability experiments were repeated and showed very similar results, i.e. the plasmid was stably maintained for more than 30 doublings. After this some plasmid loss was seen, but even after 50 generations >90% of the cells still maintained the plasmid.

Strain development through targeted gene alterations
Two recombinant FZ45 strains had been constructed previously that had shown repeatedly good titers and near theoretical yields of 110% (g succinic / g glucose). The plasmids over-expressing the two endogenous genes were introduced into the currently used *A. succinogenes* isolate, a strain that performs better in CMBP medium. Vial fermentations with the recombinant strains did not shown significant yield improvements. 4L fermentations under metabolic flux (MFA) conditions showed a 10% yield improvement. FZ45 had shown a 14 % increase in yield under identical conditions. We also tested the performance of two recombinant strains in CMBP medium. The results are summarized in Table 1.

Table 1: Yield improvements in recombinant FZ. Shown are the moles of succinic acid produced per 100 moles glucose substrate in fermentations run under flux analysis conditions (black, ID111606, 111506, 111406), see Materials and Methods, or in CMBP production medium (blue, ID 121806, 121906, 122006)).

Construct	**FZ/pvec**	**FZ/ pISTONS**	**FZ/pREB830.60**
Relevant features	vector	A. succinogenes pathway gene	A. succinogenes redox gene
Succinic acid produced [moles]	**131**	**151**	**145**
SA yields [wt%]	**85**	**96**	**93**
% yield increase	**ref**	**13%**	**9%**
Succinic Titer [g/L]	**61.6**	**64.5**	**67.0**
SA yields [wt%]	**96**	**110**	**109**
% yield increase	**ref**	**15%**	**14%**
Succinic Titer [g/L]	**64.2**	**74.6**	**75.0**

Economic modeling of the fermentations using recombinant FZ strains projected a reduction in manufacturing costs of 2.6 cents/lbs succinic acid from the increased yield and titer.

Isolation of a novel hyper-acid producer
Rumen samples were procured from a fresh, road-kill deer and from a slaughterhouse, bovine steer. Samples were ground and stored. Enrichment strategies based on substrate utilization ability were considered and sources were contacted to procure feedstocks.

Our first enrichment was run using CMBP medium, since our initial experiments under Task A had shown that CMBP can be a challenging environment. A closely related strain to FZ45, a different 130z derivative, showed greatly altered propensities for succinic production in CMBP medium. Xylose, arabinose or glycerol was used as carbon sources and rumen contents from

steer and deer were used. Frozen glycerol stocks were prepared on a regular basis, once specific product formation was demonstrated.

In all, six enrichment cultures from rumen contents were run over the course of six months as continuous fermentations, all in a CO_2 atmosphere on the various carbon sources. Three different media were tested for deer and steer rumen samples as inocula. Continuous cultures from bovine rumen enriched for specimen producing predominantly succinic acid, acetic acid and butyrate, or propionic acid depending on the carbon source. Deer rumen inocula led mainly to enrichments for 1,3 propanediol, acetate and ethanol producers. All cow rumen cultures appeared to produce more homogeneous cultures as judged by the metabolite profiles. The profiles are summarized in Table 2.

Table 2: Enrichment cultures from rumen. Steer and deer rumen were grown as described in materials and methods. Ionophoric antibiotics were added as part of the enrichment scheme for organic acid producers. Abbreviations are: corn mill by-product (CMBP), Middlebrook medium (Mb), xylose (xyl), glycerol (gly). RCCPM is a mineral medium described in materials and methods. Products were analyzed by organic acid HPLC and identified as succinic acid (SA), 1,3- propanediol (13P), propionic acid (PA), acetate (Ace), butyric acid (BA), lactic acid (LA), ethanol (EtOH).

Culture	Media	Rumen Source	Major Peaks
204-5	CMBP, Xylose	Cow	SA, Ace, BA
224-10	CMBP, Xylose	Cow	SA, BA
224-14	CMBP, Xylose	Cow	SA, Ace, BA
224-25	CMBP, Xylose	Cow	SA, Ace, BA
302-5	CMBP, Arabinose	Cow	BA
302-16	CMBP, Arabinose	Cow	PA
302-20	CMBP, Arabinose, Las	Cow	Ace, PA
302-24	CMBP, Arabinose, Las	Cow	Ace, PA
302-31	CMBP, Arabinose, Gly	Cow	13P, PA
302-37	CMBP, Arabinose, Gly	Cow	13P, PA
302-39	CMBP, Arabinose, Gly	Cow	13P, PA
324-7	TS, Gly	White Tail Deer	Ace, 13P, EtOH
324-14	TS, Gly	White Tail Deer	SA, 13P, EtOH
324-20	TS, Gly	White Tail Deer	LA, 13P
324-22	TS, Gly	White Tail Deer	SA, 13P
324-24	TS, Gly	White Tail Deer	SA, 13P, BA
324-32	TS, Gly	Cow	SA, EtOH
502-7	Mb, Gly	Cow	SA
502-8	Mb, Gly	Cow	PA
601-4	RCCPM	Cow	13P
601-10	RCCPM	Cow	13P
601-16	RCCPM	Cow	13P, BA
601-24	RCCPM	Cow	SA
601-28	RCCPM, LA, Xylose	Cow	SA

Frozen glycerol stocks have been prepared from all enrichment schemes, but isolation and identification of a specific strain was not possible.

2.3.4 Conclusions

We screened a collection of 130Z derivatives for a strain that was capable of succinic production in CMBP medium. A different isolate was identified as the best available strain in our collection. It produces higher amounts of the by-product acetate, and we speculated that this by-product leads to more favorable energetics. Acetate production is associated with the generation of reducing equivalents. Introduction of a plasmid that re-routes carbon and allows generation of additional reducing power led to enhanced succinic production in all media tested.

Enrichment cultures established good stock sources for the isolation of potentially novel organisms with interesting features. Bovine rumen samples generated cleaner cultures, i.e. enriched for those producing only one major product compared to deer rumen. However, none of these cultures achieved levels comparable to our current *A. succinogenes* isolate.

2.4 Task C Investigation of a downstream recovery process

2.4.1 Introduction

The replacement of petroleum derived chemicals such as maleic anhydride by bio-based succinic acid needs to be economical to gain market entry. The recovery of succinic acid from an aqueous fermentation broth has been shown to contribute greatly to the overall production costs. MBI has developed a fermentation process for the production of succinic acid using *Actinobacillus succinogenes* and has focused on two approaches for recovering and isolating succinic acid from fermentation broth, direct acidification and ion exchange.

Direct acidification represents the simplest approach for converting the succinate back to succinic acid. The disadvantage of this approach is that the succinic acid must then be separated from the salt by-products formed along with the other impurities from the fermentation. The use of sulfuric acid to acidify the fermentation, while more accepted in industry, generally yields salts that are less soluble, making the separation of the salt by-product from the succinic acid more problematic. The complete direct acidification process involves first clarifying the broth to remove suspended solids; the broth is then acidified and concentrated to a level that will allow the maximum amount of succinic acid to crystallize without precipitating large amounts of magnesium sulfate, typically 22% by weight magnesium sulfate. The succinic acid is collected and then re-crystallized.

MBI has previously investigated a cation ion exchange process where the succinate salt is converted to succinic acid in an ion exchange column. In this procedure the broth is first clarified to remove suspended solids and then it is passed through a proton charged strong acid ion exchange column, converting the succinate salt to free succinic acid. The succinic acid solution is passed through a carbon column to remove color impurities followed by crystallization.

The advantage of the direct acidification process is that it avoids the added capital and operation expenses of the ion exchange column, but the yield is limited by the presence of large amount of inorganic salt and requires two crystallization steps to reach a chemical purity similar to what is obtained from the ion exchange.

While MBI has investigated both of these processes to recover succinic acid from fermentation broth, there has not been a side by side comparison of the recovery approaches. None of the processes has been fully optimized using the current fermentation process and media. Data from previous pilot scale recoveries was incorporated into the economic analyses of production costs, but included assumptions based on yield and purity improvements along with assumptions for the performance of other processing steps such as membrane filtration performance and ion exchange efficiencies. These assumptions needed to be substantiated.

The goal of this work was to assess feasibility of various methods to enhance recovery of succinic acid and to evaluate the performance of a recovery process at a pilot plant scale. To accomplish this, the performance of direct acidification and ion exchange processes including the membrane filtration step were investigated at a lab scale using the current fermentation process and media. Two pilot plant scale fermentations were then performed and processed using the ion exchange process.

2.4.2 Materials and Methods

HPLC analysis: Succinic acid concentrations were determined by reverse phase high pressure liquid chromatography (HPLC) using either a Waters 1515 Isocratic pump with a Waters 717 Auto sampler and a Waters 2414 refractive index detector, with the system controlled, and data collected and processed using Waters Breeze software (version 3.3), or an Agilent 1200 series Quaternary HPLC system equipped with an auto inject and RI detector running Agilent EZChrom Elite software. For both systems a Bio-Rad Aminex HPX-87H (300mm x 7.8mm) column was used with a Cation H guard column held at 55°C and the detector was set at 35°C. The mobile phase was 0.021 N sulfuric acid running at 0.5 ml/min. Samples were filter through a 0.45 μm filter, and 5.0 μl was injected onto the column.

Internal Magnesium analysis was performed using a Varian SpectrAA-20 Plus, atomic absorption spectrometer. The integrated Automated AA Analysis software was used to develop a five point calibration curve from 0 to 25 ppm and obtain results. Atomic absorption reading were obtained in triplicate and averaged.

General Mineral analysis was performed by the Diagnostic Center for Population and Animal Health at Michigan State University. Samples were analyzed for: aluminum, antimony, arsenic, barium, boron, cadmium, calcium, chromium, cobalt, copper, iron, lead, magnesium, manganese, mercury, molybdenum, phosphorus, potassium, selenium, sodium, sulfur, thallium, and zinc by ICP-AES .

Chemical purity of succinic acid: One gram of succinic acid was accurately weighed into a 25 ml volumetric flask using an analytical balance. The sample was then dissolved in deionized water, and analyzed by HPLC. The purity was determined by dividing concentration measured by the HPLC by the concentration calculated based on the weight and dilution.

Fermentations: Standard fermentations were performed as described for Task A.

Fermentation 214 (pilot scale): Was performed in a Sartorius D150L fermentor equipped with 2 7.5 inch diameter Rushton type impeller. The fermentor was controlled through Sartorius' digital control system. Media components and fermentation conditions were scaled from the standard fermentation described in Task A, with the addition of protein hydrolysate.

Fermentation 320 (pilot scale): Was performed in a Sartorius D100L fermentor equipped with 2 6.5 inch diameter Rushton type impeller. The fermentor was controlled through Sartorius' digital control system. Media components and fermentation conditions were scaled directly from the standard fermentation described in Task A, with the addition of protein hydrolysate.

Broth clarification by Centrifugation: The broth was clarified in 500 ml centrifuge bottles using a Sorvall RC6 centrifuge. Samples were centrifuged at 10,000 x g for 15 minutes at 4^{o}C and the supernatant was decanted. Solids were suspended in a minimum of DI water and re-centrifuged for 15 minutes at 10,000 g (RCF) for 15 minutes. The supernatant from this wash was combined with the original supernatant.

Membrane filtration: Lab scale development of membrane filtration was performed on a Spectrum Laboratories KrosFlo Research II pump system equipped with the digital pressure monitor. Spectrum Laboratories polyether sulfone (PES) hollow fiber membrane filters with 1mm lumens, 65 cm^2 area and 0.2μm pore size were used (cat # X22E-301-02N). Retentate flow rate, and permeate flow were varied to determine the best conditions for the filtration.

Pilot Scale Membrane Filtration: The pilot scale fermentation broths were filtered using an Amicon Filter system. The broth was charged to a 80 gallon stainless steel kettle. The broth was withdrawn from the bottom drain, pumped through a Spectrum PES hollow fiber. The retentate was recycled to the top of the kettle and the permeate was collected in a 50 gallon Nalgene container. Broth was pumped through the filter. The filtration was started with an inlet pressure of 15 and an outlet pressure of 8, giving a transmembrane pressure (TMP) of 11.5. The TMP was slowly increased to a maximum of 14.5 psi during the run to maintain a minimum flux rate. The broth was concentrated and the retentate was then diluted with DI water while the filtration was continued.

Ion exchange-laboratory: Glass columns 75mm x 61cm, fitted with a glass frit (ChemGlass # CG-1193-31) were charged with 2.5 liters of 'Dowex GCR-S(H) cation' strong acid cation exchange resin. The broth was passed through the resin from top to bottom. The pH of the effluent from the column was measured and broth was collected until the pH began to rise. The remaining broth was collected separately as 'partially exchanged broth', which was reprocessed through the ion exchange column after it had been regenerated. The resin was regenerated by passage of several bed volumes of H_2SO_4 through the column from bottom to top. The column was then washed with DI water until the effluent was neutral. The ion exchanged broth was stirred with 100 ml of fresh resin, if necessary, to reduce the amount of residual magnesium to less than 0.01 equivalents compared to succinic acid.

Ion exchange-pilot: An eight foot tall plastic column was assembled from 6 inch schedule 80 cPVC pipe and 6 inch Harvel clear ridged PVC schedule 40 pipe, which had an internal volume of approximately 40 liters. The column was charged with 40 liters of cation exchange resin that had been allowed to soak in DI water for 24 hours. Broth was processed through the column from top to bottom. The pH of the effluent from the column was measured and broth was collected until the pH began to rise. The broth remaining in the column was collected separately as 'partially exchanged broth'. The column was regenerated and the partially exchanged broth was processed through the column prior to addition of untreated broth. The resin was regenerated by passage of several bed volumes of H_2SO_4 through the column from bottom to top. The column was then washed with DI water until the effluent was neutral. Passage of broth and regeneration of the column was repeated until all the broth had been processed

through the column. The column required four regeneration cycles for fermentation 214, and seven regeneration cycles for fermentation 320.

Carbon treatment-laboratory: After passage through the ion exchange column the broth was passed through a 25mm x 46 cm glass column slurry packed with activated carbon, mixed with 50 g of Celite. The broth was passed through the column and collected. The carbon was rinsed with 2 bed volumes, which was combined with the original material.

Carbon treatment-pilot: A five foot tall plastic column was assembled from 4 inch Harvel clear ridged PVC schedule 40 pipe. The column was charged with activated carbon. The column was washed with DI water, alternating between forward and reverse flow until the effluent ran clear. The ion exchange treated broth was passed through the column. The effluent was collected until a faint color was observed. Residual broth was then rinsed out of the column and the depleted carbon replaced with fresh.

Concentration and Crystallization-laboratory scale: Processed broth was concentrated under reduced pressure, 27" Hg, on a Buchi roto-vap. The sample was heated until a solution was obtained. The sample was cooled to 4°C to crystallize the succinic acid, which was collected by vacuum filtration and dried in a vacuum oven at 60°C.

Concentration and Crystallization-pilot: Solutions were concentrated under vacuum (20" Hg at 75°C) using a shell and tube evaporator (Whitlock). Sigma Antifoam 289, Ivanhoe Industries XFO-10SA was added to minimize foaming. Solutions were concentrated, and then drained from the evaporator into a 5 gallon plastic bucket. The mixture was slowly cooled to 4°C over two days. Crystals were collected by vacuum filtration and dried in a vacuum oven at 60°C.

Direct acidifcation procedure: Concentrated H_2SO_4 was added to clarified fermentation broth. The acidified solution was analyzed for Mg^{2+} and concentrated under reduced pressure. The mixture was cooled to 4°C and the crude crystals were collected by vacuum filtration. The crude crystals were dissolved in DI water and filtered through a carbon column. The decolorized solution was concentrated and crystals collected as previously described.

2.4.3 Results and Discussion

Ion exchange versus direct acidification

To directly compare the ion exchange and direct acidification methods, succinic acid was isolated from several fermentation broths in parallel. For these experiments the clarified broth was divided into two equal portions and a portion was processed using the ion exchange and direct acidification methods. The results of these experiments are summarized in Table 3. In addition to comparing the IEX and DA process, we also evaluated the effect of different complex nutrient sources in the fermentation on the purity of succinic acid recovered.

Table 3: Succinic acid yield and purity from lab scale recovery using IEX and DA processes

		IEX Process			DA Process			
Fermentation	Complex Nutrient Source	Purity (%)	Yield (%)	Sulfur (ppm)	Purity (%)	Yield (%)	Sulfur (ppm)	
523	Whole Liquid Feed Syrup	96.4	66.7		99.0	39.6		Note 1
526	Whole Liquid Feed Syrup	95.4	30.2		98.7	68.0		Note 2
604	Whole Liquid Feed Syrup	96.9	66.3	39.0	96.7	67.1	804.0	-
524	Clarified Liquid Feed Syrup	97.5	49.7	61.0	98.6	38.0	37.0	Note 1
603	Clarified Liquid Feed Syrup	96.6	77.2	103.0	98.8	58.7	61.0	-
629	Clarified Liquid Feed Syrup	95.6	74.9	383.0	96.9	67.9	1520.0	
614	Clarified Liquid Feed Syrup	98.5	79.7	257.0	96.6	58.0	342.0	
816	CMBP	97.9	62.1	232.0	96.5	54.0	4880.0	
927	CMBP	97.7	61.4	86.0	96.9	42.0	3390.0	Note 1

Note 1 - Operational problems led to a loss of product and reduced yield in the DA process

Note 2 - Operational problems led to a loss of product and reduced the yield in the IEX process

The purity of succinic acid recovered in all the experiments was similar regardless of the nutrient source used in the fermentation, or the method used to recover the succinic acid.

The yield from the processes varied considerably even when experiments with explainable losses are excluded from the averages. The average yield from the IEX process was higher at 70 ± 7% compared to 62 ± 6% for the DA process. It was apparent that for both processes the crystallization step(s) accounts for the largest loss, thus the lower yield with the DA process is consistent with the requirement of two crystallization steps. In particularly, the recovery from the first crystallization in the DA process is restricted by the large amount of $MgSO_4$ present.

In addition to the yield and purity, samples were also submitted for general mineral analysis. While this gave ppm levels of a variety of different elements, of particular interest to us was the level of sulfur in the samples. A target market for succinic acid production could be conversion to butanediol (BDO), which is accomplished by catalytic hydrogenation. The catalyst used for this conversion is sensitive to compounds containing reduced sulfur, thus the level of sulfur in the recovered succinic acid is important to its suitability for conversion to BDO. The amounts of sulfur contained in the samples are listed in Table 3. The sulfur level in succinic acid recovered by the IEX process varied from 39 to 383 ppm, whereas the sulfur in succinic acid recovered from by the DA process varied from 37 to 8040 ppm. The larger variability in the sulfur in succinic acid recovered by the DA process may have been due to residual magnesium sulfate in the samples. Since sulfate is not a poison to hydrogenation catalyst this would not be as problematic as a reduced sulfur species. Attempts to have the samples analyzed for sulfate were unsuccessful since the succinic acid interfered with the sulfate analysis.

In these experiments the IEX and DA process gave similar results, however neither process was optimized. While it is expected that optimization would improve yields for both processes, the yield from the DA process will always be limited by the first crystallization. In the DA process, to prevent co-crystallization of large amounts of $MgSO_4$ with the succinic acid, the maximum amount the broth can be concentrated is limited by the amount and solubility of the $MgSO_4$. This limits the maximum theoretical recovery possible from the first crystallization in the DA process. The IEX process is not subject to this limitation since the salt is removed by ion exchange, suggesting that the recoveries from an optimized IEX process should be higher than for an optimized DA process. In addition since the sulfur level in the IEX process was lower than for the DA process it was decided to use the IEX for the pilot plant validation runs.

Process validation

To confirm recover process performance two small pilot scale fermentation where completed and the broth processed using the IEX recovery process. The recoveries are given in Table 2.

Table 4: Percent recoveries of succinic acid for pilot scale validation batches.

	Percent recovery succinic acid					
	Microfiltration	IEX	Carbon	Concentration	Crystallization/collection*	Overall*
Fermentation 214	95%	99%	94%	90%	83%	67%
Fermentation 320	106%	93%	96%	99%	87%	82%
Average	101%	96%	95%	94%	85%	74%
Model/target	**97.50%**	**99%**	**99%**	**99%**	**97%**	**92%**

An obvious error is the 106 % recovery for the microfiltration in fermentation 320. Most likely, this is due to an error in the second succinic acid analysis, which led to an over estimate of the amount of succinic acid obtained from the microfiltration, and resulted in a comparatively low yield in the ion exchange step. However we were unable to confirm this retroactively.

Overall, fermentation 214 recovered 4.2 Kg of succinic acid with 99.6 % chemical purity using the ion exchange process. Fermentation 320 recovered 4.0 Kg of succinic acid that was 98.4 % chemically pure.

The average overall yield for the pilot batches was within the range obtained in lab experiments, but below what had previously been entered in the economic model. Two recoverable sources of losses were identified. First, some reduction in yield was due to hold up in the processing equipment, particularly for fermentation 214. This material accounted for 4% of the overall reduction in yield and was recovered when the equipment was washed. It is not expected that a commercial operation would suffer this loss, since it would not be necessary to wash the processing equipment between batches. The second recoverable loss was during the crystallization. Succinic acid has a solubility of 30 g/l at 4°C however HPLC analysis of the mother liquors indicated higher values. This difference accounts for a 3.3 % increased loss in the mother liquor. Although observed as an increased loss to the mother liquor, the apparent increased solubility is due to incomplete cation exchange, during the ion exchange step. This was confirmed by atomic absorption analysis of the Mg content in the mother liquor. In addition to excess succinate contained in the mother liquor, the crystallization step also had a poor mass balance. Only 94 % of the product was recovered as product, in the mother liquor, or in the equipment washes. A poor mass balance was also seen for the carbon treatment, where only 96 % of the product was accounted for. If the lost product was recovered through improved operation the overall average yield would improve to a value of 81 %. To reach the targeted recovery the unexplained losses/poor mass balance obtained for the carbon treatment and the crystallization step will have to be identified and eliminated.

In addition to validating the yield and purity of the product recovered by this process, the amount sulfuric acid used to regenerate ion exchange columns was assessed. Our pilot scale fermentations used considerably more acid than expected based on our calculations. Some operational factors led to the increase in the amount for sulfuric acid required. The efficiency of the regeneration is also limited by the chemical properties of the resin and sulfuric acid. The selectivity of the resin for Mg^{2+} varies depending on the crosslink density of the resin, which is not the case for H^{+}. Thus the equilibrium between Mg^{2+} and H^{+} favors having the Mg^{2+} bound to the resin, thereby increasing the amount of sulfuric acid required to regenerate the resin. The

second factor affecting the efficiency of the regeneration step is the relative pKa's of sulfuric acid. Only the first proton on sulfuric acid is strongly acidic and efficient at regenerating the resin. The pKa of the second proton on sulfuric acid is 2, and is only about 10 % ionized, making it inefficient at regenerating the ion exchange resin. While optimization of the regeneration procedure could reduce the required amount of sulfuric acid, depending on the non-ideality of the system, but it would not be possible to reach the target projected in the model without greatly increasing the size of the cation exchange columns. This would lead to increased capital and operational cost.

The amount of carbon used was measured for fermentation 320, and although it may be possible to regenerate the carbon, this was not evaluated.

While the lab scale results suggested that the ion exchange process was better, the pilot plant results revealed several costs for the ion exchange process that are likely to be much higher than anticipated, such as the amount of acid required to regenerate the cation exchange resin, and the amount of carbon required. This, in addition to the lower than anticipated yield, warrants a re-evaluation/comparison of the direct acidification recovery process.

Membrane Filtration

Clarification of the fermentation broth is the first step in the recovery processes. Both centrifugation and membrane filtration were considered. However, while centrifugation did remove most suspended solids the resulting broth was cloudy, whereas membrane filtration resulted in a clear broth. Membrane filtration was evaluated by MBI both internally and by an outside contractor (GEA filtration). The evaluation internally used polyether sulfone (PES) membranes and provided clarified broth for other recovery work, in addition to providing data on membrane performance. Operating conditions were identified that increased the flux rate through the membrane from 5 l/m^2-hr to 25 l/m^2-hr through the PES membrane.

To test other membrane material, specifically stainless steel and ceramic, 600 liters of broth was shipped to GEA Filtration, who tested the broth using, polymeric, ceramic, and stainless steel membranes. A copy of their report is included as Appendix A. In their testing the stainless steel membranes performed best and they achieved higher than targeted flux rates, which makes membrane filtration a viable option for clarification of succinic acid fermentation broth on a commercial scale.

2.4.4 Conclusion

Although the laboratory investigation suggested that the ion exchange recovery process was the better recovery process, pilot scale validation of the ion exchange process revealed several deficiencies that will lead to increased production costs over what had been modeled. We were not able to achieve the desired yield for the process, nor did the column regeneration and carbon treatment perform as anticipated. In addition, potential commercial users were interested in less expensive crude material over a more costly purified product. All of these factors led us to re-evaluate the ion exchange versus direct acidification process.

3.0 References

Booth, I.R., Ferguson, G.P., Miller, S., Li, C., Gunasekera, B, and Kinghorn, S. 2003, Biochemical Society Transactions **31**, 1406-1408.

Guettler, M.V., Jain, M.K., Rumler, D. 1996, U.S. Patent 5,573,931.

Guettler, M.V., Jain, M.K., Soni, B.K. 1998, U.S.Patent 5,723,322 .

Guettler, M.V., Rumler, D., Jain, M.K. 1999, *Internat. Journ. Systematic Bacteriology* **49**, 207-216.

Hong, S.H., Kim, J.S., Lee, S.Y., In, Y.H., Choi, S.S., Rih, J.-K, Kim, C.H., Jeong, H., Hur, C.G., Kim, J.J. 2004, *Nature Biotechnol.* **22**, 1275-1281.

Jablonski, P.E., M. Jaworski, and C. J. Hovde 1996, FEMS Microbiol Lett **140**:165-9.

Lee, P.C., Lee, S.Y., Hong, S.H. and Chang, H.N. 2002, *Appl. Microbiol. Biotechnol.* **58**, 663-668.

McKinley, J.B., Zeikus, J.G., Vieille, C. 2005, *Appl. Environmental Microbiol*. **71**, 6651-6656.

Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Plainview, NY, 1990.

Van der Werf, M. J., Guettler, M. V., Jain, M. K., and Zeikus, J. G. 1997, Arch. Microbiol. **167**: 332-342.

4.0 Products Developed

Kleff, S. Presentation at the World Congress for Industrial Biotechnology, March 2007, Orlando, FL ; *The Integrated Bio-Refinery: Conversion of Corn Fiber toValue-added Chemicals.*

Kleff, S. Presentation at the DOE Peer Review, August 2007, Denver, CO; *Succinic Acid as a Byproduct of Corn-Based Ethanol Biorefineries.*

Kleff, S., Teymouri,F., Guettler, M.V., Hanchar, R. J., Saffron, C., 29th Symposium in Biotechnology for Fuels and Chemcials, May 2007, Denver, CO; *The Integrated Bio-refinery: Conversion of Corn Fiber to Value-Added Chemicals.*